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Abstract

Jellyfish, a promising source of bioactive compounds, have attracted the attention of the biotechnology sector. This research has explored the antioxidant and antimutagenic properties, genotoxicity, and stability of peptides derived from blue cannon jellyfish (*Stomolophus* sp. 2) collagen hydrolysates (JCH) as potential food supplements. JCH was fractionated into three parts: F1 (molecular weight > 10 kDa), F2 (10 kDa > molecular weight > 3 kDa), and F3 (molecular weight < 3 kDa) using ultrafiltration. Notably, F3 exhibited the highest antioxidant and antimutagenic activities while remaining non-genotoxic, supporting its safety for potential applications. The function of JCH and its fractions to quench free radicals persisted even after three months at 4°C. In addition, *in silico* analysis revealed 15 unique peptides in *Stomolophus* sp. 2 collagens, ten of which showed particularly promising bioactive potential. Peptides from *Stomolophus* sp. 2, with molecular weight under three kDa, exhibit remarkable bioactivity, holding great promise for future research into bioactive food supplements.

Keywords: bioactive peptides; *in silico* analysis; jellyfish hydrolysates; genotoxicity; storage; ultrafiltration

1. Introduction

The rapid worldwide increase of jellyfish has led to a highly advantageous fishery and an unconventional source for traditional fishermen, mainly owing to the decay of conventional fishing resources and their affordable cost [1]. This decay has considerably affected the traditional fishermen's financial stability, forcing them to face severe economic difficulties [2]. Conversely, jellyfish fisheries represent a substantial opportunity for improving the economic conditions of these fishermen [3], especially with the production of bioactive peptides derived from jellyfish collagens [4].

According to systematic studies of jellyfish species, the valid species within the jellyfish family in the American Continent are *Stomolophus meleagris* [5] and *Stomolophus fritillaria* [6]. In the southern Gulf of California, specifically in the Upper Gulf of California, there resides an as-yet-undefined species of *Stomolophus* jellyfish, known as *Stomolophus* sp. 2, commonly referred to as the blue cannonball jellyfish [7]. *Stomolophus* sp. 2 offers considerable potential profit for those highly dependent on fishing activities [3]. Moreover, the jellyfish harvesting has become an economic alternative for coastal fishermen in central and southern Sonora, with a production about 57,000 tons reported in 2024 [8]. Their extracted collagen shows notable antioxidant capacity [9]. Its gelatine has

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been found to possess antimutagenic activity [10], adding to the potential health benefits of jellyfish collagen hydrolysates.

Jellyfish collagen hydrolysates hold significant promise as novel sources of bioactive peptides, offering a range of health benefits such as antioxidants, antihypertensives, antimicrobials, and antiproliferative effects [11]. These antioxidant peptides, typically composed of 2 to 20 amino acids and with molecular weights below 3,000 Da, have been the focus of increasing scientific interest [12]. Conversely, jellyfish enzymatic hydrolysis produces peptides with molecular weights greater than 8,000 Da [13]. The molecular weight of marine protein hydrolysates is a crucial factor in obtaining protein hydrolysates with bioactive peptides [14].

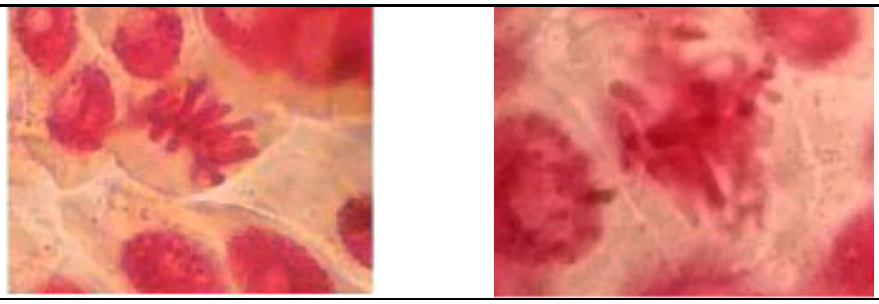
An alternative method for generating peptides with specific molecular weights is membrane separation, a technique widely used in industry due to its economical nature. The ultrafiltration membrane separation process has been shown to enhance the specific bioactivity of jellyfish hydrolysates [15–17]. However, despite these research efforts, there is a significant lack of information about the application of ultrafiltration to improve the antioxidant and antimutagenic properties of *Stomolophus* sp. 2 mesoglea collagen hydrolysates. This gap in knowledge underscores the urgency and importance of our research. Furthermore, according to our understanding, the antimutagenic activity and potential genotoxic effects of *Stomolophus* sp. 2 collagen hydrolysates have not been investigated following ultrafiltration, further highlighting the need for our study.

The present work considers blue cannonball jellyfish (*Stomolophus* sp.2) mesoglea as a source of collagen. It aims to document the bioactivity (antioxidant and antimutagenic), genotoxicity, and antioxidant storage stability over time of the fractions obtained by ultrafiltration after enzymatic hydrolysis of jellyfish collagen with Alcalase. Additionally, the bioactive peptides encoded in *Stomolophus* sp. 2 mesoglea's collagen proteomes were predicted *in silico*.

2. Results

2.1. Collagen and Hydrolysates Yield and Properties

The percentage of collagen after extraction, estimated from its hydroxyproline content (5.7 ± 0.38 g/100 g), was 60.3 ± 2.2 % with a DH value of 27.2 ± 1.3 %. The hydrolysate yield (expressed as grams of dry hydrolysates per 100 g of collagen) was 11.08 %. These hydrolysates at 100 ppm show a low adverse effect on the chromosomes (3.4 % of abnormalities). Different phases of normal and abnormal mitosis are shown in **Figure 1**. Based on these images, it was determined whether the hydrolysates or fraction three samples exerted harmful effects to the chromosomes, as discussed later.



(**a**) (**d**)

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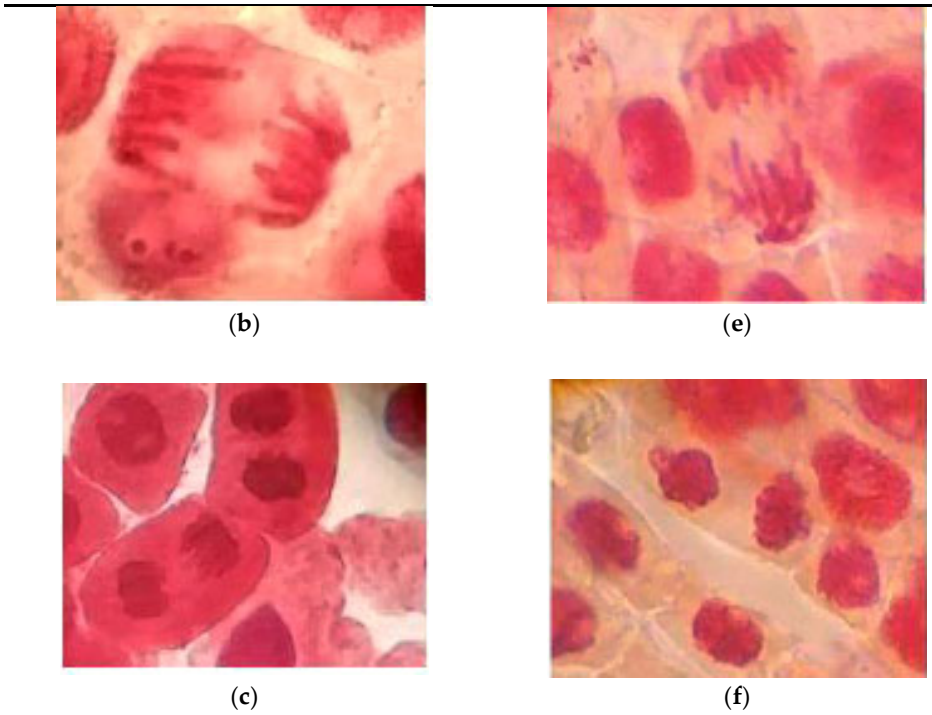

Figure 1. Root sections of Cambray onion (*Allium fistulosum*) observed under a light microscope at 100x. A) Normal metaphase; B) Normal anaphase; C) Normal telophase; D) Metaphase with chromosome breakage; E) Anaphase with chromosome breakage; F) Telophase with chromosome breakage. Negative control: water; positive control: sodium azide.

Antioxidant values (**Table 1**) showed that the obtained hydrolysates exhibited the ability to undergo single electron transfer (SET) mechanisms against the ABTS radical, as well as hydrogen atom transfer (HAT) capacity against the AAPH radical, and ferric reducing antioxidant power (FRAP). Moreover, these hydrolysates effectively inhibited the mutation induced by AFB_1 on *Salmonella typhimurium* strain TA100, with an inhibition percentage greater than 50 % (**Table 2**), which could be considered as a moderate inhibition of the control mutagen [18].

Table 1. Antioxidant activity of mesoglea jellyfish *Stomolophus meleagris* collagen hydrolysates and their fractions obtained by ultrafiltration measured by scavenging capacity of ABTS radical cation, to reduce Fe^{3+} to Fe^{2+}, (FRAP), and scavenge oxygen-derived radicals (ORAC).

Method	Sample	mmol TE/g*
ABTS	Hydrolysates	7920 ± 0.4^{b}
	F1 (>10 kDa)	5763 ± 9.0^{d}
	F2 (3-10 kDa)	6848 ± 3.1^{c}
	F3 (< 3 kDa)	8993 ± 5.2^{a}
FRAP	Hydrolysates	6435 ± 2.1^{b}
	F1 (>10 kDa)	1244 ± 1.1^{d}
	F2 (3-10 kDa)	4345 ± 3.8^{c}
	F3 (< 3 kDa)	7622 ± 5.6^{a}
ORAC	Hydrolysates	457 ± 4.3^{b}
	F1 (>10 kDa)	223 ± 2.5^{d}
	F2 (3-10 kDa)	324 ± 1.1^{c}

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	F3 (< 3 kDa)	599 ± 3.0[a]

* Data presented are the mean of three determinations (standard deviation). Mean values followed by different letters express significant differences ($p < 0.05$) among determinations and samples tested.

Table 2. Effect of mesoglea jellyfish *Stomolophus meleagris* collagen hydrolysates and its fractions obtained by ultrafiltration on the mutagenicity induced by AFB1 using *Salmonella typhimurium* TA100 assay*.

Sample	Dose (mg/plate)	Revertants	% Inhibition
Collagen	2	896 ± 3.0[f]	72.6
	0.2	1444 ± 7.0[d]	55.8
	0.02	2805 ± 3.0[b]	14.1
	0.002	3245 ± 38[a]	0.6
Hydrolysate	2	477 ± 6.0[n]	85.4
	0.2	708 ± 7.0[k]	78.3
	0.02	1323 ± 9.0[e]	59.5
	0.002	1577 ± 5.2[c]	51.7
F1 (>10 kDa)	2	464.9 ± 7.7[n]	85.8
	0.2	598.5 ± 1.0[l]	81.7
	0.02	725.2 ± 4.4[j]	77.8
	0.002	822.9 ± 3.0[h]	74.8
F2 (3-10 kDa)	2	401.3 ± 1.2[ñ]	87.7
	0.2	590.6 ± 2.2[l]	81.9
	0.02	706.2 ± 4.1[k]	78.4
	0.002	814.6 ± 1.7[i]	75.1
F3 (< 3 kDa)	2	392.5 ± 2.9[ñ]	88.0
	0.2	577.6 ± 3.1[m]	82.3
	0.02	701.5 ± 1.4[k]	78.6
	0.002	871.3 ± 3.0[g]	73.3
Negative control		3265.4 ± 26.2	
AFB_1		159.3 ± 12.9	

* Data presented are the mean ± standard deviation of three plates. Mean values followed by different letters express significant differences ($p<0.05$) among doses and samples tested.

The hydrolysates obtained were rich in glycine, glutamic acid, aspartic acid, arginine, and proline, accounting for 23, 13, 10, 9, and 9 % of the total amino acid composition, respectively. The amino acids with hydrophobic chains, glycine, alanine, valine, leucine, isoleucine, proline, phenylalanine, and methionine, represent about 49 % of jellyfish hydrolysates.

2.2. Fractions Characterization

2.2.1. Antioxidant Capacity

The membrane ultrafiltration process applied to hydrolysates allowed the separation of the peptides based on their molecular weight (F1, F2, F3). The three obtained hydrolysate peptide fractions showed the capacity to donate hydrogen atoms and electrons, reduce Fe^{3+} to Fe^{2+}, and scavenge oxygen-derived radicals (**Table 1**). Among them, the most significant antioxidant activity ($p < 0.05$) was detected in the fraction with < 3 kDa (F3). As expected, low-molecular-weight peptides exhibit higher antioxidant activity [19].

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2.2.2. Antimutagenic Capacity

The highest antimutagenic activity, measured as the percentage of inhibition of *S. typhimurium* TA100 revertants/plate, was detected in F3 even at the lowest concentration (**Table 2**), which was consistent with the antioxidant activity detected. Therefore, the peptides present in the obtained fractions may protect against the type of mutation induced by the TA100 strain, base substitutions or frameshift mutations [20].

2.2.3. Genotoxicity of Hydrolysates and Fraction < 3 kDa

The JCH and F3 did not cause a change in the frequencies of different cell stages, and their treatments did not induce a wide range of mitotic abnormalities as compared to the sodium aside in the root tip of *Allium fistulosum* (**Figure 1**). The mitotic index (MI) of *A. fistulosum* root was not significantly ($p > 0.05$) decreased by JCH and F3 (**Table 3**). The level of abnormalities and MI of JCH and F3 indicate that these compounds are not genotoxic at 100 ppm, respectively [21].

Table 3. Clastogenic effects of *Stomolophus* sp. 2 hydrolysates and fraction F3 (<3kDa MW) on the mitotic cells of *Allium fistulosum*.

Sample	Mitotic index[1,2] (%)	Abnormalities[2] (%)
Water	47.1	1
Sodium azide	43.2	47
Hydrolysates	55.7	3.4
F3	59.8	1.1

[1]Values represent the average of three repetitions. [2]The mitotic index and abnormality levels indicated that hydrolysates and F3 are not genotoxic at 100 ppm.

2.2.4. Effect of the Storage Time on the Antioxidant Activity of Hydrolysates and Fractions

The effect of storage time (90 days) at 4 °C on the ability to scavenge ABTS radicals by the hydrolysates and fractions is shown in **Figure 2**. Fraction F3 (< 3 kDa MW) remained more stable during the 90 days of storage, showing a slight decrease in its ability to quench the ABTS radical. In contrast, a gradual yet significant ($p < 0.05$) decline after 60 days of storage was observed in the hydrolysates and fractions F1 (> 10 kDa MW) and F2 (3 - 10 kDa).

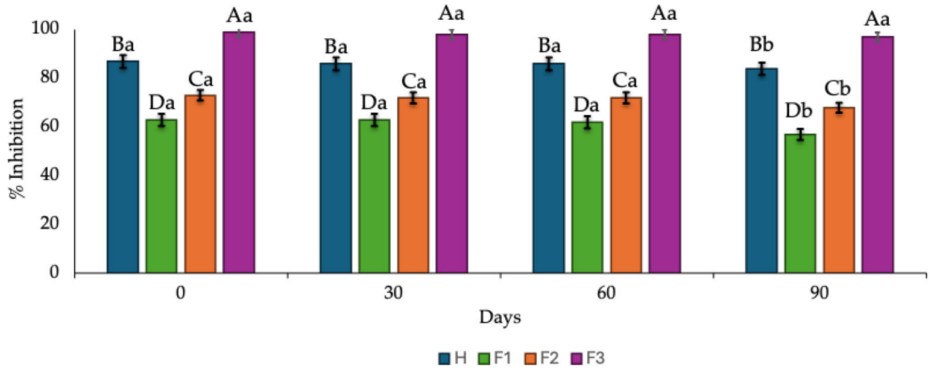

Figure 2. Effect of the storage time at 4°C on the ability to scavenge ABTS radical of hydrolysates and fractions. Capital letters indicate differences among hydrolysates and fractions ($p<0.05$). Lowercase letters denote differences due to the storage time ($p<.0.05$).

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2.2.5. Bioactive Peptides Identified by Informatics Analysis

The proteins identified from jellyfish protein extracts using nano-LC-MS/MS were derived from 3,086 identified spectra (PSM), which included 707 distinct peptides corresponding to 10 proteins. Notably, collagen was identified among the proteins. The collagen characterized in this study was type IV, encompassing 15 unique peptides (**Table 4**).

Bioactive peptides remain inactive while embedded in proteins and exhibit their activity once released through enzymatic action. In this study, the bioactive peptides in the collagenous extracts of *Stomolophus* sp. 2 mesoglea were predicted using Peptide Ranker software (http://distelldeep.ucd.ie/PeptideRanker). The analysis revealed that 100% of the identified peptides had a molecular weight below 3,000 Da, with 80% falling within the range of 1,000 – 2,215 Da and 20% below 1,000 Da. Among the 15 unique peptides identified in the collagen of *Stomolophus* sp. 2 through bioinformatic analysis, ten peptides scored above 0.5 [22,23], indicating a high probability of bioactivity (**Table 4**).

Table 4. Jellyfish collagen peptides identified by bioinformatic analysis (accession number tr|V9GWB0|V9GWB0_CRASO[1]).

Peptide identified	Average Mass[2]	Ppm[3]	Length[4]	Bioactive probability[5]
KGNEGPPGEKGL	1,181	6.9	12	0.56
KGQPGPGGSADF	1,116	6.9	12	0.62
PGQNGLRGADGIKGEPGL	1,734	9.6	18	0.63
KGAVGEPGPKGDL	1,412	7.4	14	0.62
KGEPGESGGL	929	0.4	10	0.56
GDTGLDGEKGNKGEPGARGEI	2,199	9.5	22	0.47
KGDAGTNGL	831	6.3	9	0.42
AGVEGPPGPPGF	1,080	6.1	12	0.80
GPPGDQGPQGL	1,219	0.7	12	0.84
GSQGPTGEKGANGLPGL	1,708	1.1	18	0.78
PPGDQGPQGL	964	6.6	10	0.77
GNAGPKGEPGESGGL	1,495	2.3	16	0.63
PRGDPGQKGEPGQ	1,420	5.2	14	0.25
KGARGLNGTGGEKGSRGPRGF	2,215	6.4	22	0.50
GRDGAGVKGNAGPKGEPGESGGL	2,179	3.9	24	0.36

[1]Accession number according to PEAK STUDIO 8.5 database. [2] Theoretical masses of identified peptides retrieved from database. [3] Absolute mass error; for values ≤ 10 ppm, the potential for false positives is considered low [24]. [4] Theoretical lengths of identified peptides retrieved from database. Scale: 0.0: highly unlikely to be bioactive; > 0.5: high probability of bioactivity; 1.0: highly likely to be bioactive [22,23].

3. Discussion

It is considered that hydroxyproline content is the best means for estimating the percentage of collagen. In the present work, the collagen content detected in *Stomolophus* sp. 2 was comparable to values previously reported for another jellyfish species, as *Rhopilema esculentum* (65%) [25] and *Rhopilema pulmo* (61.15%) [26].

The *in vitro* Ames antimutagenic assay is a biological assay that uses a mutant strain of *Salmonella typhimurium* to evaluate whether a substance can prevent or reduce the mutagenicity induced by a mutagenic agent. Since cancer is often linked to DNA damage, the test also serves as a rapid assay to estimate the anticarcinogenic potential of a compound. If the compound is antimutagenic, it reduces the frequency of mutations (revertants) observed in the bacteria compared to the control, indicating that the substance possesses protective properties against genetic damage [27]. The Ames test showed that the *Stomolopus* sp. 2's hydrolysate contained peptides with antimutagenic effects, capable of inhibiting mutations or DNA damage associated with carcinogenesis. These peptides present in the

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hydrolysates could act as antioxidants and cell regulators, making them a promising alternative for disease prevention. However, more studies are required to confirm this.

The antioxidant and antimutagenic activities detected in the obtained hydrolysates could be attributed to the presence of some amino acids, such as glycine and proline. Glycine acts as an indirect antioxidant by being a component of glutathione, a potent antioxidant in the body. It also has antimutagenic properties by suppressing the formation of free radicals and stabilizing cell membranes, thus helping to protect against cellular damage and oxidative stress [28]. Proline, on the other hand, can act as a compatible osmolyte to protect cells, and reduces oxidative damage caused by environmental factors and antimutagenic agents in various organisms [29].

In vitro assays assess the ability of a group of compounds to interact with a free neutral radical that possesses an unpaired electron in one of its orbitals [30]. Free radicals are unstable and highly reactive; when they seek stability through electron uptake, they can initiate chain reactions that damage cellular structures, including membranes, lipids, and proteins [31]. Therefore, the peptides present in the fractions obtained from collagen hydrolysates extracted from jellyfish, by presenting antioxidant properties, may help to protect cells present in organisms against oxidative stress by scavenging free radicals and enhancing natural antioxidant defenses. It has been shown that peptides extracted from jellyfish collagen can increase the levels of superoxide dismutase [12], a key cellular antioxidant, and reduce reactive oxygen species [32].

The higher antioxidant and antimutagenic capacity of the fraction with molecular weight below 3 kDa may be attributed to its exposure to a hydrophobic surface during the fractionation process [33]. This fraction could have more amino acids exposed to the surface, enhancing their interaction with free radicals, converting them into more stable products [33] protecting the cells against oxidative stress more efficiently. However, an amino acid profile of each fraction should be developed to confirm this. Moreover, the peptides' efficiency in reacting with radicals as electron donors and preventing radical chain reactions is strongly affected by their amino acid sequence.

The stability of peptides is considered crucial to establishing their application as functional ingredients [34]. The results obtained in this study indicated that both the hydrolysates and fractions F1 and F2 maintained more than 80% of their antioxidant activity, while fraction F3 retained almost 100% of this activity. The slight decrease detected in the antioxidant capacity of the hydrolysates and fractions F1 and F2 can be attributed to peptide modifications such as dehydration, glycation, and oxidation of aromatic rings, which alter their structure and consequently their bioactivity [35]. The apparent greater stability of fraction F3 could be attributed to the type of secondary structure formed by its peptides, as well as to the sequence of its amino acids [36]. It has been documented that the peptides present in hydrolyzed proteins can form both b-sheet and a-helix structures, with the b-structure being the most stable [36]. Likewise, if amino acids such as aspartic acid, glutamic acid or arginine are found in the peptide sequence, peptides will be more susceptible to moisture absorption [37].

Previous research has indicated that jellyfish collagen is chemically simple, contributing to its versatile and adaptable tissue properties. As a result, it has been classified as collagen type 0 [38]. This type of collagen exhibits a chemical composition similar to that of various collagen classes [38] and shares numerous functions with more specialized collagens [39], including antioxidant properties [11], through the Peptide Ranker program and the ranks assigned to the probability of specific peptide of being bioactive [22]. The eight peptides identified with a substantial likelihood of bioactivity, containing fewer than 20 residues with the presence of amino acids considered crucial for the antioxidant activities of peptides [40]: glycine, proline, leucine, alanine, phenylalanine, and valine. Therefore, the predicted peptides present in the *Stomolophus* sp.2 collagen could be responsible for the bioactive properties detected in the obtained hydrolysates and fractions. Nevertheless, to ensure this, studies integrating *in vitro* and *in silico* techniques are necessary to evaluate and confirm their potential antioxidant and antimutagenic activity.

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4. Materials and Methods

4.1. Samples and Chemicals

Sixty-five jellyfish *Stomolophus sp. 2* specimens from Kino Bay (28°43'N/111°54'W, 24-36°C) were collected. The jellyfish organisms' measures were weight 0.45 to 1.1 kg and length 12-17 cm. Specimens stored in an ice bed system were transported to the laboratory, and mesoglea was gathered from the organisms. All chemicals used were of analytical reagent grade and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

4.2. Collagen Extraction

The extraction of collagen followed the procedure described previously [9]. Mesoglea jellyfish were cut into small pieces (100 g), and collagen was obtained with 0.1 M NaOH (1:5 w/v) and mechanically stirred for 24 h. After centrifugation (39,200 x g, 4 °C, 90 min), the pellet was sequentially rinsed until the pH dropped to seven and freeze-dried. After that, it was treated with 0.5 M CH_3COOH (1:5 w/v) and pepsin (10 mg/sample in 0.5 M CH_3COOH; 1:5 w/v). At each step, after stirring for 24 h, the samples were centrifuged (39,200 x g, 4 °C, 15 min). Finally, the samples dialyzed at 4 °C against water (cellulose membrane 50 kDa molecular weight cut-off) were lyophilized and stored at -80°C for further analyses. The collagen present in the sample was determined by its hydroxyproline (Hyp) and protein content. Crude protein was quantified with a LECO FP-2000 Nitrogen Protein Analyzer (Method 9993.13, AOAC. 2000. "Official Methods of Analysis" 17th ed. Association of Analytical Chemists. Washington, DC.). Hyp was determined by high-performance liquid chromatography (RP–HPLC, Agilent Technologies) [41].

4.3. Preparation of Enzymatic Hydrolysates

Hydrolysates were produced by a commercial enzyme system (Alcalase) according to the method described previously [42] with some modifications. The pepsin solubilized collagen (200 mg) was dissolved in a 100 mM sodium phosphate buffer of pH 7.5 (0.4 mg protein/mL). The beakers were placed in a 55 °C water bath under constant mixing for 5 h. The enzyme-substrate ratio (E/S) was 0.2 % (w/w). The enzyme was inactivated by heating the sample to 95 °C for 15 min. The supernatants obtained after centrifugation (6000 x *g*, 15 min) were considered to contain hydrolysates (JCH). The freeze-dried samples were stored at -80 °C for further assays.

4.4. Membrane Ultrafiltration

The JCH was dissolved in deionized water (1 mg/mL) and fractionated by ultrafiltration through an ultrafilter (Amicon Stirred Cell, Model 8200) equipped with a 10 kDa membrane into one fraction: F1, composed of peptides with molecular weights >10 kDa, The F3 was subjected to ultrafiltration using a 3 kDa membrane and fractionated into two fractions: F2 consisting of peptides from molecular weight >3 kDa and <10 kDa, and F3, comprising peptides of molecular weight <3 kDa. The fractionated temperature was 4 °C, and the pressure was 0.2 MPa. The collected fractions were freeze-dried and stored at -80 °C.

4.5. Analysis

4.5.1. Degree of Hydrolysis (DH)

DH was established by analysing free amino groups by reaction with the *O*-phthaldialdehyde (OPA) reagent [43]. The serine was employed as a standard. The a, b, and htot values were 1.00, 0.40, and 8.6, respectively.

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4.5.2. Amino Acid Profile of Hydrolysates

The amino acid content of JCH was determined by reverse-phase high-performance liquid chromatography (Hewlett-Packard RP-HPLC, Agilent Technologies Inc., Santa Clara, CA, USA). Freeze-dried samples (100 mg) were hydrolysed under reduced pressure (6 M HCl, 150°C, 6 h). The hydrolysate residues, after centrifugation, were neutralised in 2 mL of 4 M NaOH and filtered using a cellulose-acetate syringe filter unit (0.2 mm). After filtration, the samples were mixed with potassium borate buffer (pH 10.4) and *O*-phthaldialdehyde (1:1, v/v) and applied to the RP-HPLC system. The chromatogram recordings and integrations were performed using ChemStation software (Agilent Technologies). Fluorescence was measured at wavelengths of 330 nm (excitation) and 418 nm (emission) [44]. Analysis was performed in triplicate, and the results were expressed as g/100 g sample.

4.5.3. Antioxidant Activity of Hydrolysates and Fractions

Three assays evaluated the *in vitro* antioxidant activity of JCH and its fractions at a concentration of 0.75 mg/mL, and the results were expressed as mmol TE (Trolox equivalent)/g.

The sample scavenging capacity to reduce the 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) was measured [45]. ABTS in water (7 mmol) was dissolved in 2.45 mmol potassium persulfate (dark room, 25 °C, 16 h) to produce the $ABTS^{\bullet+}$. Then, 20 µL aliquot samples were added to 980 µL of $ABTS^{\bullet+}$ diluted with methanol (Abs_{734nm} = 0.70), and the decrease in absorbance was measured at 734 nm using a 96-well microplate reader (Thermo Fisher Scientific Inc.).

The ferric reducing antioxidant power (FRAP) capacity of the samples was adapted to microplate equipment and carried out according to previous studies [46]. The FRAP working solutions comprised of 300 mM acetic acid–sodium acetate buffer (pH 3.6), 20 mM FeCl3•6 H2O, and 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl (10:1:1 ratio). The mixture of FRAP working solution (280 mL) and the sample (20 mL) was incubated at 25°C in the dark for 30 min before its absorbance was recorded at 638 nm using a microplate reader.

Samples' capacity to quench the oxygen radicals produced by the 2,2′-azobos(2-amidinopropane) (AAPH) was evaluated by the ORAC assay [47], which was the third chemical assay. The inhibition of fluorescence decay was evaluated over 60 min at 37°C at 485 nm (excitation) and 520 nm (emission) in a Cary Eclipse spectrophotometer (Agilent Technologies) after mixing 100 mL of the samples with a mixture of 75 mM phosphate buffer, pH 7.3 (1.7 mL), 0.36M AAPH (0.1 mL), and 0.048 mM fluorescein (100 mL).

4.5.4. Antimutagenic Activity of Hydrolysates and Fractions

Salmonella/microsome assay [48] was employed to evaluate the antimutagenic potential of hydrolysates and fractions. Salmonella tester strain TA100 was used with bioactivation (S9, Aroclor 1254-induced, Sprague-Dawley male rat liver in 0.154 M KCl solution). Bacteria reproduction (2×109 cells/ml, overnight culture, 37 °C) was developed in a nutrient broth. The mutagen employed was Aflatoxin B_1 (with S9 mix) (500 ng/mL). The assay involved 100 μL of hydrolysates (0, 0.5, and 5 mg/mL) and fractions (0, 0.002, 0.02, 0. 2, and 2.0 mg/mL) in test tubes. Then, each tube was mixed with bacteriological agar containing histidine and biotin, bacterial culture (100 μL), and S9 mix (500 μL). 10% DMSO (100 µL) without AFB_1 was used as a negative control. The mixture obtained was transferred to minimal agar plates. The plates were incubated (37 °C, 48 h), and each plate's revertant bacterial colonies were counted. The inhibition rate for mutagenic activity was calculated according to Equation 1.

$$Inhibtion\ Rate\ (\%) = \left[\frac{(1-T)}{M}\right] \times 100 \quad Equation\ 1 \tag{1}$$

where T is the number of revertant in presence of AFB_1 and test samples plates, and M is the number of revertant per plate in the mutagen without extras, subtracting the spontaneous revertant from the numerator and denominator. A > 40% was considered as strong antigenotoxicity, 25–40% moderate

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antigenotoxicity, and ≤ 25% no antigenotoxicity (Owen et al., 2004). Each dose was tested in triplicate.

4.5.6. Storage Antioxidant Stability of Hydrolysates and Fractions

For three months, freeze-dried hydrolysates and fractions (about 10 mg) were placed in Eppendorf tubes and stored in a 4°C refrigerator to explore their stability and ability to scavenge the radical ABTS.

4.5.5. Genotoxicity Test of Hydrolysate and Fraction 3

Onions (*Allium cepa*) were allowed to germinate by immersion in distilled water and stored in the dark at 25 ± 2°C. Onions roots about 5 cm long were used for testing. The onion roots were treated with hydrolysate and F3 samples at 50 and 100 ppm concentrations for 24 h. The control group was treated with distilled water. The root tips were dehydrated (45 min) in a 3:1 (v/v) ethanol–acetic acid solution, then fixed in 1 N hydrochloric acid (2 min, 60 °C). After that, the samples were stained with orcein for one minute and finally squashed. The mitotic cells were observed and counted with a microscope.

4.5.7. Nano LC-MS/MS Analysis

The collagen sample was dissolved and identified with a nano LC-MS/MS platform (Ultimate 3000 nano-UHPLC system and Orbitrap Q Exactive HF mass spectrometer with Nanospray Flex Ion Source, Thermo Scientific) as previously reported [10]. The pepsin protein extracts were poured into a C18 SPE column (Thermo Scientific) with 0.1% formic acid to remove the salt, and 1 mg of the sample was loaded into the Nanoflow UPLC. The MS/MS conditions were set as follows: scan (300 – 1,650 m/z, resolution 60,000 at 200 m/z, 3e6; operated in Top 20 mode (resolution 15,000 at 200 m/z; automatic gain control target 1e5; maximum injection time 19 ms; normalized collision energy 28%; and an isolation window of 1.4 Th); and charge state exclusion parameters set to unassigned, 1, > 6, and a dynamic exclusion of 30 s. Raw MS files were analysed and searched against the jellyfish protein database based on the species of the samples using PEAKS Studio 8.5. The parameters were set as follows: the protein modifications were carbamidomethylation (fixed) and methionine oxidation (variable), and the enzyme specificity was pepsin. There were two maximum missed cleavages; the precursor and ion mass tolerance were 10 ppm and 0.5 Da, respectively.

4.6. Statistical Analysis

The statistical design applied to the chemical characterization of the jellyfish collagen hydrolysates and fractions was used to minimize variation in the replicates. Data (n = 3) obtained from the biological activities were subjected to the ANOVA method ($p < 0.05$) to investigate differences in variance using the SPSS® program (SPSS Statistical Software, Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation out of three determinations. Significant differences ($p < 0.05$) between the results were identified using the Tukey test.

5. Conclusions

The hydrolysates derived from jellyfish collagen demonstrated notable in vitro antioxidant and antimutagenic properties while showing no clastogenic effects. Following the ultrafiltration process, the antioxidant and antimutagenic activities of the hydrolysates were enhanced; however, clastogenicity remained unaffected. The highest bioactivity observed in fractions with a molecular weight of less than 3.0 kDa is likely attributed to the presence of peptides that aid in scavenging free radicals and blocking reactive oxygen species. These findings indicate that low molecular weight hydrolysate peptide fractions from jellyfish collagen warrant further investigation for purification and potential application as a bioactive food supplement.

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Abbreviations

The following abbreviations are used in this manuscript:

JCH	Jellyfish collagen hydrolysates
F1	Fraction molecular weight > 10 kDa
F2	Fraction 10 kDa > molecular weight > 3 kDA
F3	Fraction molecular weight > 3 kDa
ABTS	2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulfonic-acid)
FRAP	Ferric reducing antioxidant power
AAPH	2,2′-azobis(2-methylpropionamidine) dihydrochloride
FRAP	Ferric reducing antioxidant power
SET	Single electron transfer
HAT	Hydrogen atom transfer
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
UHPLC	Ultra-High-performance liquid chromatography
ANOVA	Analysis of Variance
ORAC	Oxygen radical absorbance capacity

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